MARKED-UP SPECIFICATION

Of those tested, ODNs shorter than 8 bases were non-stimulatory (e.g., Table 1, ODN 4e). Among the forty-eight 8 base ODN tested, a highly stimulatory sequence was identified as TCAACGTT (SEQ.ID.NO:90) (ODN4) which contains the self complementary "palindrome" AACGTT (SEQ.ID.NO:105). In further optimizing this motif, it was found that ODN containing Gs at both ends showed increased stimulation, particularly if the ODN were rendered nuclease resistant by phosphorothioate modification of the terminal internucleotide linkages. ODN 1585 (["5' GGGGTCAACGTTGACGGGGG 3'] GGGGTCAACGTTGAGGGGGG (SEQ ID NO: 12)), in which the first two and last five internucleotide linkages are phosphorothioate modified caused an average 25.4 fold increase in mouse spleen cell proliferation compared to an average 3.2 fold increase in proliferation included by ODN 1638, which has the same sequence as ODN 1585 except that the 10 Gs at the two ends are replaced by 10 As. The effect of the G-rich ends is *cis*; addition of an ODN with poly G ends but no CpG motif to cells along with 1638 gave no increased proliferation. For nucleic acid molecules longer than 8 base pairs, non-palindromic motifs containing an unmethylated CpG were found to be more immunostimulatory.

results are shown in Table 11.

Effective ODNs began with a TC or TG at the 5′ end, however, this requirement was not mandatory. ODNs with internal CpG motifs (*e.g.*, ODN 1840) are generally less potent stimulators than those in which a GTCGCT (SEQ. ID. NO: 58) motif immediately follows the 5′ TC (*e.g.*, ODN 1967 and 1968). ODN 1968, which has a second GTCGTT (SEQ. ID. NO: 57) motif in its 3′ half, was consistently more stimulatory than ODN 1967, which lacks this second motif. ODN 1967, however, was slightly more potent than ODN 1968 in experiments 1 and 3, but not in experiment 2. ODN 2005, which has a third GTCGTT (SEQ. ID. NO: 57) motif, inducing slightly higher NK activity on average than 1968. However, ODN 2006, in which the spacing between the GTCGTT (SEQ. ID. NO: 57) motifs was increased by the addition of two Ts between each motif, was superior to ODN 2005 and to ODN 2007, in which only one of the motifs had the additional of the spacing two Ts. The minimal acceptable spacing between CpG motifs is one nucleotide as long as the ODN has two pyrimidines (preferably T) at the 3′ end (*e.g.*, ODN 2015). Surprisingly, joining two GTCGTT (SEQ. ID. NO: 57) motifs end to end with a 5′ T also created a reasonably strong inducer of NK activity (*e.g.*, ODN 2016). The choice of thymine (T) separating consecutive CpG dinucleotides is not absolute, since ODN 2002 induced appreciable NK activation despite the fact that adenine (A) separated its CpGs (*i.e.*, CGACGTT; SEQ. ID. NO: 113). It should also be noted that ODNs containing no CpG (*e.g.*, ODN 1982), runs of CpGs, or CpGs in bad sequence contents (*e.g.*, ODN 2010) had no stimulatory effect on NK activation.

Table 10

ODN	Sequence (5'-3')	LU	
cells alone		0.01	
1754	ACCATGGACGATCTGTTTCCCCTC	0.02	SEQ ID NO: 59
1758	TCTCCCAGCGTGCGCCAT	0.05	SEQ ID NO: 45
1761	TACCGCGTGCGACCCTCT	0.05	SEQ ID NO: 60
1776	ACCATGGACGAACTGTTTCCCCTC	0.03	SEQ ID NO: 61
1777	ACCATGGACGAGCTGTTTCCCCTC	0.05	SEQ ID NO: 62
1778	ACCATGGACGACCTGTTTCCCCTC	0.01	SEQ ID NO: 63
1779	ACCATGGACGTACTGTTTCCCCTC	0.02	SEQ ID NO: 64
1780	ACCATGGACGGTCTGTTTCCCCTC	0.29	SEQ ID NO: 65
1781	ACCATGGACGTTCTGTTTCCCCTC	0.38	SEQ ID NO: 66
1823	GCATGACGTTGAGCT	0.08	SEQ ID NO: 6
1824	CACGTTGAGGGGCAT	0.01	SEQ ID NO: 67
1825	CTGCTGAGACTGGAG	0.01	SEQ ID NO: 68
1828	TCAGCGTGCGCC	0.01	SEQ ID NO: 69
1829	ATGACGTTCCTGACGTT	0.42	SEQ ID NO: 70
1830[2]	RANDOM SEQUENCE	0.25	
1834	TCTCCCAGCGGGCGCAT	0.00	SEQ ID NO: 71
1836	TCTCCCAGCGCGCGCCAT	0.46	SEQ ID NO: 72
1840	TCCATGTCGTTCCTGTCGTT	2.70	SEQ ID NO: 73
1841	TCCATAGCGTTCCTAGCGTT	1.45	SEQ ID NO: 74
1842	TCGTCGCTGTCTCCGCTTCTT	0.06	SEQ ID NO: 75
1851	TCCTGACGTTCCTGACGTT	2.32	SEQ ID NO: 76

Table 15. Specific blockade of CpG-induced TNF-α and IL-12 expression by inhibitors of endosomal acidification or NFκB activation

			Inhibitors:									
activators	Medium		Bafilomycin (250 nM)		Chloroquine (2.5 μg/ml)		Monensin (10 μM)		NAC (50 mM)	TPCK (50 μM)	Gliotoxin (0.1 μg/ml)	Bisgliotxin (0.1 μg/ml)
	TNF-α	IL-12	TNF-α	IL-12	TNF-α	IL-12	TNF-α	IL-12	TNF-α	TNF-α	TNF-α	TNF-α
Medium	37	147	46	102	27	20	22	73	10	24	17	41
CpG ODN	455	17,114	71	116	28	6	49	777	54	23	31	441
LPS	901	22,485	1370	4051	1025	12418	491	4796	417	46	178	1120

Table 15 legend IL-12 and TNF-α assays: The murine monocyte cell line J774 ($1x10^5$ cells/ml for IL-12 or $1x10^6$ cells/ml for TNF-α), were cultured with or without the indicated inhibitors at the concentrations shown for 2 hr and then stimulated with the CpG oligodeoxynucleotide (ODN) 1826 (TCCATGACGTTCCTGACGTT SEQ ID NO: 10) at 2μM or LPS (10 μg/ml) for 4 hr (TNF-α) or 24 hr (IL-12) at which time the supernatant was harvested. ELISA for IL-12 or TNF-α (pg/ml) was performed on the supernatants essentially as described (A. K. Krieg, A.-K. Yi, S. Matson, T. J. Waldschmidt, G. A. Bishop, R. Teasdale, G. Koretzky and D. Klinman, *Nature* 374, 546 (1995); Yi, A.-K., D. M. Klinman, T. L. Martin, S. Matson and A. M. Krieg, *J. Immunol.*, 157, 5394-5402 (1996); Krieg, A. M, *J. Lab. Clin. Med.*, 128, 128-133 (1996). Cells cultured with ODN that lacked CpG motifs did not induce cytokine secretion. Similar specific inhibition of CpG responses was seen with IL-6 assays, and in experiments using primary spleen cells or the B cell lines CH12.LX and WEHI-231. 2.5 μg/ml of chloroquine is equivalent to< 5 μM. Other inhibitors of NF-κB activation including PDTC and calpain inhibitors I and II gave similar results to the inhibitors shown. The results shown are representative of those obtained in ten different experiments.

Excessive immune activation by CpG motifs may contribute to the pathogenesis of the autoimmune disease systemic lupus erythematosus, which is associated with elevated levels of circulating hypomethylated CpG DNA. Chloroquine and related antimalarial compounds are effective therapeutic agents for the treatment of systemic lupus erythematosus and some other autoimmune diseases, although their mechanism of action has been obscure. Our demonstration of the ability of extremely low concentrations of chloroquine to specifically